Preparation and Charact rization of a β-Lactamase–Fab′ Conjugate for th Site-Specific Activation of Oncolytic Agents

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Antibody-directed catalysis (ADC) is a two-step method for the targeted delivery of chemotherapeutic agents in which enzyme–antibody conjugates, prelocalized to antigen-bearing cells, activate prodrugs designed to be substrates for the enzyme. An enzyme–Fab′ conjugate exhibiting both native β-lactamase activity and immunoreactivity toward carcinoembryonic antigen (CEA) was constructed. Treatment of CEA-expressing LS174T cells with this conjugate imparted β-lactamase activity to the cells; β-lactamase activity was not imparted by treatment with unconjugated β-lactamase and not to CEA negative cells treated with conjugate. Cephalosporin-based prodrugs, and other substrates synthesized as model compounds, were found to have wide variations in their kinetic parameters toward the conjugate, with k_{cat} values ranging from 16 to 3300 s^{-1} and K_M values ranging from 5 to 160 μM. The prodrug derived from desacetylvinblastine-3-carboxylic acid hydrazide (DAVLBHYD) was studied in vitro and found to be 5-fold less cytotoxic to LS174T cells than the parent DAVLBHYD. For antigen-positive cells preincubated with conjugate, however, the prodrug showed the same potency as the parent drug. Thus, the combination of conjugate and prodrug appears to provide antigen-dependent toxicity to tumor cells.

EXH. I

INTRODUCTION

Antibody-directed catalysis (ADC[1]) is a form of noncovalent drug delivery in which an enzyme–antibody conjugate is localized to a tumor target, where it converts subsequently administered prodrug to active drug. The ADC approach has been adopted as one way of overcoming potential difficulties with the delivery of therapeutic levels of free drugs to tumor targets using covalent protein-based delivery systems.

Demonstration of accumulation and retention of radiolabeled haptens by prelocalized bifunctional antibodies showed that the latter act as artificial receptors and proved that targeting of small molecules in vivo does not require covalent linkage to the targeting molecule. Furthermore, these studies showed that two-step noncovalent delivery has advantages for certain applications over covalent delivery. For instance, bifunctional antibody-based noncovalent delivery of isotopically labeled haptens results in decreased radiation exposure of normal tissues relative to covalent delivery (*1*). ADC represents an extension of this system in which the synthetic receptor not only binds the ligand but also activates and releases it. A number of enzyme–prodrug systems have been described for the antibody targeted activation of prodrugs (*2–9*).

Of the multitude of enzymatic reactions which could potentially be used to activate prodrugs, the most directly and generally applicable appeared to be the type in which a molecule is cleaved into two pieces, especially that subset in which the enzyme recognizes only a portion of the substrate and is relatively insensitive to the identity of the remainder. Reactions catalyzed by β-galactosidase, exopeptidases, and various phosphatases are among those for which substrate recognition depends predominantly on one portion of the molecule and is independent of the remainder. However, a disadvantage of these enzymes is that they each cleave only a small number of linkage types, e.g. acetal, peptide, phosphate ester, which limits the variety of drugs that can be used. Ideally, the enzyme of choice should be capable of cleaving substrates connected through a wide range of functional groups, providing the ability to deliver several drugs with a single enzyme–antibody conjugate.

In addition, enzymatic activity that does not occur in eucaryotic organisms is advantageous for clinical use because it avoids interference from endogenous enzymes in nontarget tissues (activity which may be induced by the prodrug), or from endogenous substrates, inhibitors, etc. Other factors affecting enzyme selection included requirements for diffusable cofactors, molecular weight, and stability. β-Lactamase meets all of these criteria and, in addition, is relatively easy to obtain and purify. The β-lactamase produced by the P99 strain of *Enterobacter cloacae*, is particularly attractive since it can be produced as a substantial fraction of the cell's total protein (*10, 11*), has good cephalosporinase activity (*12*) and is relatively insensitive to substrate side-chain modification. The amino acid sequence for this enzyme has been determined, both by amino acid analysis and by gene sequencing (*13*), and the enzyme has been crystallized (*14*), though a high-resolution crystal structure has not yet been published.

β-Lactamase can cause the release of many different substituents from the 3′ position of cephalosporins (*15*), depending on their leaving group propensity. This is because the r lease occurs in a reaction that is secondary

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[1] Abbreviations: ADC, antibody-directed catalysis; CEA, carcinoembryonic antigen; DAVLBHYD, desacetylvinblastine-3-carboxylic acid hydrazide; β-l, β-lactamase; β-lCEM, β-lactamase–CEM231 Fab′ 1:1 conjugate; sulfo-SMCC, sulfosuccinimido 4-(*N*-malimidomethyl)cyclohexane-1-carboxylate; BBS, 50 mM sodium borate, 100 mM sodium chloride (pH 8.2), containing 0.01% NP40 (Pierce); NP40, Nonidet P40; NHS, *N*-hydroxysuccinimide; DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; RIA, radioimmunoassay; PADAC 2-[(*N*,*N*-dimethylanilin-4-yl)azo]pyridinium 3′-cephalosporin; PBS, 15 mM sodium phosphate, 100 mM NaCl (pH 7.4), containing 0.01% NP40; EBSS-MEM, Eagle basic salt solution–minimal essential media.

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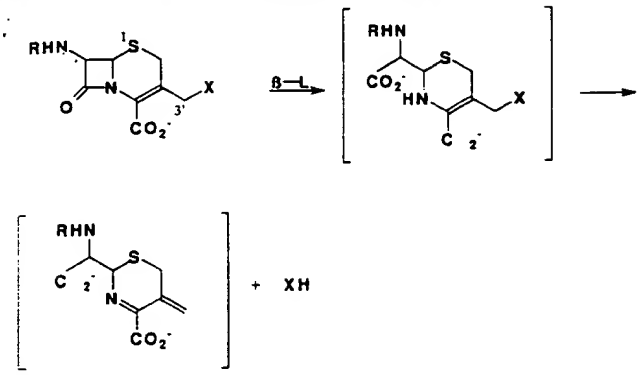


Figure 1. β-Lactamase catalyzes the hydrolysis of the cephalosporin β-lactam ring. Expulsion occurs subsequent to ring opening depending on the leaving group propensity of the 3′ substituent.

to β-lactam ring hydrolysis (*16*, *17*), the reaction actually catalyzed by the enzyme (Figure 1). Consequently, the linkage between cephem and drug is not directly acted upon by the enzyme and the 3′ substituent generally does not impede catalysis. Advantage has been taken of the 3′ substituent release upon ring opening to design dual-action antibiotics which act primarily as cephalosporins but release a secondary antibiotic when cleaved by a β-lactamase in the target bacteria (*18*, *19*).

One of the goals of the ADC system was to make use of the carcinoembryonic antigen CEA. Potential barriers preventing the effective use of CEA for immunochemotherapy, such as low or heterogeneous antigen expression and shedding of the antigen into interstitial space, could be circumvented by the amplifying properties of enzyme targeting and the ultimate release of a readily diffusable drug. CEA has been the target in many preclinical antibody–drug conjugate studies (*20*–*24*), including a few in which bifunctional-antibody-mediated noncovalent localization of vinca alkaloids to tumors provided an enhanced therapeutic effect (*25*–*27*).

The conjugation methodology is derived from procedures described by Yoshitake et al. (*28*). The heterobifunctional cross-linker sulfo-SMCC (Pierce) covalently connects lysine ε-amino groups on the β-lactamase to naturally occurring sulfhydryls in the hinge region of the antibody Fab′ fragment. This provides a heterogeneous conjugate that is regiospecific with respect to Fab′, is comparable in immunoreactivity and enzymatic activity to the proteins from which it is derived, and has a known molecular weight as small or smaller than Fab'_2 fragments.

This paper describes the conjugation procedure, properties of the conjugates, and kinetic parameters obtained with selected substrates. A detailed description of prodrug syntheses is in preparation. Behavior of the system in nude mouse models is under investigation.

EXPERIMENTAL PROCEDURES

Syntheses of LY262319, LY262594, and LY266070 will be reported elsewhere. The synthesis of LY191026 has been described (*9*). All were shown to be analytically pure by HPLC, and structures were identified by NMR and MS before being tested as substrates. LY223425 (*29*) and DAVLBHYD (*30*) were generous gifts from G. Cullinan of Lilly Research Laboratories. Their preparation has been described. Keflin (cephalothin) was the gift of B. Jackson of Lilly Research Laboratories and is commercially available (Sigma).

D rivatizati n of β-Lactamase with Sulfo-SMCC. β-Lactamase was purified according to the method of Cartwright (*31*) after isolation from cell paste by a combination of freeze–thaw and sonication. The purified enzyme was dialyzed into BBS and concentrated to 10 mg/mL. Its concentration was determined by A_{280}, assuming $A_{280}^{1\%} = 14$ (*32*). To 2 mL of the protein solution was added an aqueous solution containing 2.0 molar equiv of sulfo-SMCC (Pierce).

The concentration of this reagent was determined by measuring the release of NHS in a basic aqueous solution (70 mM Na_2CO_3). The molarity of the active ester was calculated by measuring the rise in OD_{260} using $\Delta\epsilon_{260} = 8 \times 10^3$.

The β-lactamase–SMCC reaction mixture was allowed to incubate at room temperature for 1 h, unstirred. The reaction mixture was then applied to a 1.5 × ~23 cm P6DG (Bio-Rad) column and eluted with 50 mM ammonium citrate, 100 mM sodium chloride, 1 mM DTPA (pH 6.3). The peak was collected manually in a single fraction. The concentration of protein in the peak fraction was measured by A_{280} again and was typically between 2.5 and 4.0 mg/mL. The average number of maleimide residues per enzyme molecule was determined by cysteine titration and quantitation of unreacted cysteine with DTNB (Aldrich).

A typical maleimide determination used 10 nmol of β-lactamase and 20 nmol of cysteine in a 500-μL reaction volume. Cysteine oxidation in the absence of maleimide was also determined as a control. The maleimide to β-lactamase ratio was typically 0.8–1.0 under the conditions described.

Preparation of Fab′. CEM-231 (murine IgG1) Fab'_2 fragments were prepared from intact antibody, which has been shown to react with CEA (*33*), by digestion with 3% pepsin (pepsin:Ab) (Boehringer Mannheim) at pH 4.1 in 0.1 M NaOAc, 0.1 M NaCl, at 37 °C for 4–6 h. The digestion was terminated by neutralization, followed by dialysis in 0.1 M sodium borate, 0.1 M NaCl (pH 8.2).

The Fab'_2 fragments were reduced to Fab′ with 20 mM cysteine, in the presence of 1 mM DTPA, at 37 °C for 10 min. SDS PAGE was used to confirm that these conditions give complete hinge-region reduction with minimal heavy–light chain reduction. Cysteine was removed by P6DG (Bio-Rad) gel filtration in 50 mM ammonium citrate, 0.1 M NaCl, 0.5 mM DTPA (pH 6.3). DTNB assay showed between 2 and 3 reactive sulfhydryls per fragment.

Parent antibody and antibody fragment concentrations were determined by A_{280} assuming $A_{280}^{1\%} = 14$.

Conjugation of β-Lactamase to Fab′. To the maleimide-β-lactamase solution was added Fab′ at a concentration of either 1.0 or 1.5 molar equiv of Fab′ per maleimide (see results). After a 1-h incubation at room temperature, the reaction was stopped by addition of a 10-fold excess of *N*-ethylmaleimide as a 1 M ethanolic solution. The mixture was then applied to a 2 × 100 cm Sephadex G-150 superfine gel filtration column and eluted with BBS. Critical fractions were analyzed by PAGE and then pooled according to purity of the desired molecular weight component. The conjugate was then concentrated by ultrafiltration to 2–4 mg/mL for storage and diluted to the desired concentration for use. Purity of the conjugate was determined by SDS PAGE and by gel permeation HPLC, using two 0.9 × 30 cm Zorbax 250 columns (Du Pont) in series, eluted with 100 mM sodium phosphate (pH 7.0), at a flow rate of 1 mL/min.

Immunoreactivity of the β-Lactamase–Fab′ C njugat . Immunoreactivity of the conjugates, with reactant antibody and Fab′ as control, was measured in a solid-phase assay as described by Phelps et al. (*34*). Conjugate binding was also checked in a competitive binding assay. Serial 2-fold dilutions of antibody from a starting con-

centration of 3.3 μM were prepared in DMEM + 10% fetal calf serum. To 10^6 LS174T cells were added 50 μL of the above dilution and 50 μL of 100 μg/mL CEM 231-FITC. Cells were incubated with antibody for 1 h at 4 °C and then washed three times and fixed in 1% paraformaldehyde. Fluorescence was determined by flow cytometry and is reported as linear equivalent relative fluorescent intensity.

Enzymatic Activity of the β-Lactamase–Fab′ Conjugate. β-Lactamase activity was measured by monitoring the change in absorbance of the chromogenic substrate PADAC (Calbiochem) at 570 nm in a stirred cuvette at 37 °C in PBS. Absorbance was measured every 5 s for 120 s using a Hewlett-Packard 8451A spectrophotometer, and linear portions of the rate plots were used to obtain reaction velocities. K_M and k_{cat} were determined from the slope and intercept of Lineweaver–Burk plots of the velocity data. PADAC's ϵ_{570} was taken to be 4.8×10^4 (product label).

Because of PADAC's low solubility and high extinction coefficient, the assay was run under conditions in which the rate is dependent on the substrate concentration. Consequently, the runs comparing activity between different preparations were all performed with a starting $A_{570} \approx 0.5$.

Substrate Kinetic Parameters. Kinetic parameters for conjugate acting on Keflin, LY262319, and LY262594 were measured as for PADAC, except that the change in absorbance was monitored at around 260 nm. LY262319 and LY262594 have a residual absorbance after hydrolysis, so $\Delta\epsilon$'s were obtained by completely hydrolyzing a known concentration of the substrate and calculating $\Delta\epsilon$ from the change in absorbance: $\Delta\epsilon_{258}$ for LYS262319 = 6.60×10^3 (cm M)$^{-1}$; $\Delta\epsilon_{260}$ for LY262594 = 8.4×10^3 (cm M)$^{-1}$.

The spectral change on β-lactamase-catalyzed hydrolysis of prodrug substrates LY191026 and LY266070 was too small to be useful for quantitative determination of the rates. Consequently, HPLC methods were developed to monitor these reactions. Vials containing 1.5-mL solutions of varying concentrations of substrate at 37 °C in PBS (pH 7.4) were treated with 0.11 nM β-lCEM. Samples were quenched after 90 s by adding 0.5 mL of reaction solution to 0.5 mL of 34% acetonitrile in 200 mM potassium phosphate (pH 3.0). Control experiments were performed to determine appropriate concentrations so that less than 10% of the substrate would be consumed during the 90-s reaction, to assure a linear reaction rate. Duplicate samples of the quenched reaction mixtures were injected onto a 0.46 × 15 cm C-18 reverse-phase HPLC column eluted isocratically at 1 mL/min with the buffer containing 34% acetonitrile in 200 mM potassium phosphate. The starting prodrug and/or product concentration was monitored by absorbance at 266 nm.

Determination of Antigen-Mediated β-Lactamase Activity. Antigen-positive (LS174T) and -negative (MOLT4) tumors were harvested from nude mice. Single cell suspensions were prepared at 2×10^7 cells/mL: LS174T 23% viable, MOLT4 75% viable.[2] To 1 mL of cells was added sufficient conjugate or β-lactamase to bring the assay concentration to the desired level. Cells were incubated either 1 or 10 min and washed with PBS. The activity was measured by the difference in absorbance at 570 nm before and after a 10-min incubation with PADAC. Results were recorded as the percent change in 570-nm absorbance over the incubation period.

[2] Cell viability was not considered crucial to the successful performance of the assay.

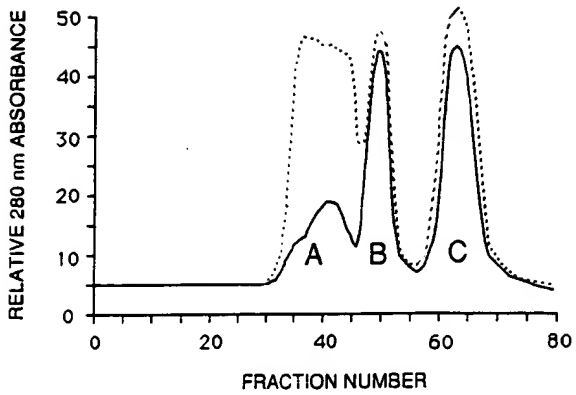


Figure 2. Purification by gel permeation chromatography o the Fab′–maleimido-β-lactamase reaction mixture. Compariso of elution patterns for 1:1 (dashed line) and 1.5:1 (solid line Fab′:maleimido-β-lactamase reaction mixtures. Ten milliliter of 15 mg/mL protein was loaded on a 2 × 100 cm column o Sephadex G-150 superfine equilibrated with BBS and elute with BBS at 0.1–0.2 mL/min. Fractions were approximately 4. mL. Peak A contains high molecular weight byproducts, E contains the 1:1 conjugate with which studies were performed and C contains unreacted β-lactamase and Fab′.

In Vitro Conjugate-Mediated Prodrug Activation Target cells resuspended in 75% leucine-deficient EBSS MEM + 10% dialyzed fetal bovine serum + gentamicir were seeded into 96-well plates (2×10^4 cells/well) anc incubated overnight. All incubations were performed a 37 °C, 5% CO_2. The supernatants were removed, anc appropriate wells treated with CEM or β-lCEM at 25 μg mL (280 nM) for 1 h. The wells were washed and 0.2 mL of the drug or prodrug at 10, 1, 0.1, 0.01, or 0.001 μg/mL vinca content were added to the cells. The plates were incubated for the desired length of time (1–48 h). At the end of the treatment period the cells were washed anc incubated in fresh media for the remainder of the 48-h period. Supernatants were then removed, 4 μCi of tritiated leucine in 0.2 mL of leucine-deficient media was added to each well, and the plates were incubated overnight. The cells were harvested, and the uptake of tritiated leucine was determined by liquid scintillation counting. All samples were run in quadruplicate.

RESULTS

The G-150 elution profile of conjugation reactions performed under two different sets of conditions (Figure 2) shows that products are formed in three molecular weight ranges. The components consist of 1:1 β-1 to Fab conjugate (peak B), along with unreacted Fab′ and β-1 (peak C), and higher molecular weight adducts (peak A). The higher molecular weight adducts are the result of either the reaction of more than one sulfhydryl in the Fab′ hinge region with a sulfo-SMCC-modified β-1 or the reaction of Fab's with more than one maleimide on a single β-1 molecule. Increasing the ratio in the reaction mixture of Fab′ to maleimido-β-lactamase from 1:1 to 1.5:1 caused an overall improvement in the yield of 1:1 conjugate. The purification also improved due to decreased peak overlap with a smaller amount of high molecular weight product.

Analytical gel filtration of the purified product (peak B, Figure 2) on Zorbax G-250 columns (broken line, Figure 3) showed that the conjugate has a molecular weight in the desired range. Molecular weights were also confirmed by polyacrylamide gel electrophoresis (not shown) comparing purified product to Fab′, β-lactamase, Fab′$_2$, and whole antibody. Immunoreactivity of the conjugate was evaluated in a whole-cell assay (Figur 4) in which binding of fluorescent-labeled whole CEM231 to LS174T cells was

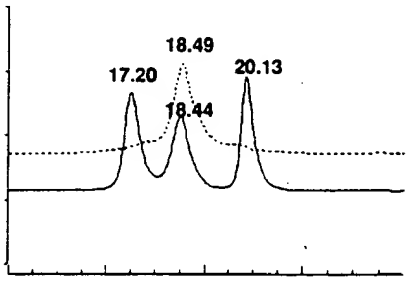


Figure 3. Analytical gel sizing HPLC profile of purified conjugate (dashed line) monitored by absorbance at 280 nm. Elution positions of known proteins (solid line): whole antibody (17.20 min) and β-l (20.13 min) mixed with 1:1 β-l–Fab′ conjugate. Analyses were performed using two 0.9 × 30 cm Zorbax 250 columns in series eluted with 100 mM sodium phosphate (pH 7.0) at 1 mL/min. Molecular weight identification was confirmed by SDS PAGE.

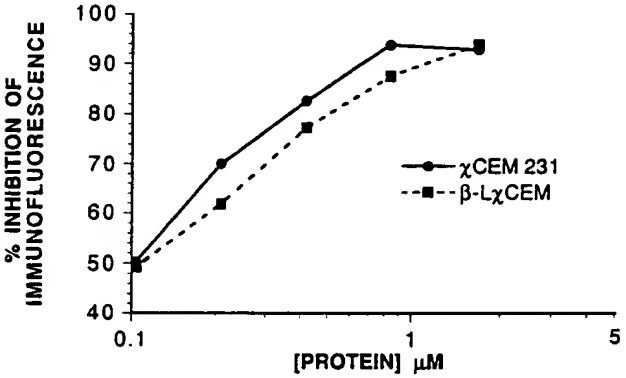


Figure 4. Competitive binding immunoreactivity comparison of parent antibody and conjugate. The ability of conjugate (dashed line) and parent antibody (solid line) to competitively inhibit the binding of FITC-labeled parent antibody to antigen-positive LS174T cells was determined as described in the Experimental Procedures.

Table I. Comparison of PADAC Kinetic Parameters for Conjugate and Enzyme

enzyme	K_M, μM	k_{cat}, s^{-1}
β-lactamase	53 ± 18[a]	106 ± 33
β-lCEM	55 ± 12	135 ± 31

[a] Standard deviations of the parameters are calculated from the standard deviations of the slope and intercept (data analyzed using the linear regression module of the RS/1 computer program, BBN Software Products Corp.) of a single Lineweaver–Burk plot. Velocities were measured at four different concentrations in either duplicate or triplicate at each, and at least 11 points were used to calculate each velocity.

competitively inhibited by conjugate, and by direct binding of iodinated conjugate to an antigen-bearing solid phase. In the whole-cell assay, conjugate immunoreactivity was comparable to that of parent antibody. In the solid-phase assay, the conjugate showed immunoreactivity of 79%, while a single binding arm bifunctional antibody was 96% immunoreactive.

Enzymatic activity of the conjugate was determined by comparing Lineweaver–Burk plots for conjugate and native enzyme using PADAC as substrate (Table I). Both K_M and k_{cat} are experimentally indistinguishable. The marginal difference in k_{cat} could be due to inexact values for the protein extinction coefficients. Modification of β-lactamase by sulfo-SMCC was found to have no effect on β-lactamase activity.

Catalytic parameters were measured for a set of available substrates in an attempt to predict the impact of drug structure on catalytic activity. K_M, k_{cat}, and their ratio were found to vary substantially (Table II) among the compounds tested. The finding that the cephem β-sulfoxides tested exhibited better hydrolytic stability than the native cephem's (see Conclusions) led us to test their activity as β-lactamase substrates. The results (Table II: LY262594 vs LY262319) show that the cephem sulfoxides can be better substrates, in terms of k_{cat}/K_M ratio, than their native cephem analogues.

Large relative errors (on the order of 50%) are associated with the k_{cat} and K_M values for PADAC, LY191026, and LY266070. In the case of PADAC and LY266070, this is because insolubility prevented velocity determinations at concentrations above about 20% of the K_M. Consequently, there are no points close to the intercept of the Lineweaver–Burk plot. Obtaining more points in the low substrate concentration range does not improve the confidence limit. This actually amounts to an inability to measure k_{cat} and K_M independently, since the slope of the plot, which represents the ratio, can be determined relatively precisely.

In the case of LY191026, the uncertainty is due to the fact that measurements of the very rapid velocity require very low substrate concentrations to remain in the linear range, and consequently, the ability to accurately integrate the HPLC peaks becomes limiting.

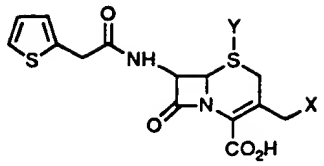
The bifunctional nature of the conjugate, i.e. its ability to simultaneously bind antigen and catalyze cephem ring opening, was tested by allowing it to bind with antigen-bearing cells, rinsing the cells to remove unbound conjugate, and then measuring the ability of cells resuspended in buffer to hydrolyze PADAC. The results (Table III) show that the conjugate has the ability to bind to cells in vitro and thereby impart β-lactamase activity. The quantity of the enzymatic activity associated with the cells depended on the time and concentration of conjugate in the incubation mixture. Treatment of antigen-positive cells with β-lactamase showed that the activity could not be attributed to nonantigen-mediated adherence of β-l to the cells. Treatment of antigen-negative cells with conjugate confirmed that interaction of conjugate with cells is mediated strictly through the antibody binding site. A background level of "activity" (5–7% ΔOD) was observed in the negative controls, attributable to dilution in the assay. The slightly higher activity observed at the high β-l level may have resulted from insufficient washing.

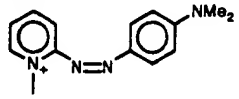
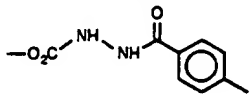
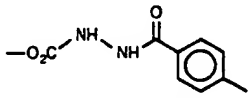
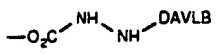
The in vitro cytotoxicity of LY266070 or DAVLBHYD incubated for various times in the presence of CEM or β-LCEM is shown in Figure 5. The cytotoxicity of DAVLBHYD is not affected significantly by incubation in the presence of CEM or β-lCEM, whereas LY266070 toxicity is greatly enhanced (lower ID_{50} values) by incubation for 1, 3, or 5 h in the presence of the conjugate. LY266070 toxicity cannot be distinguished from DAVLBHYD at the 24-h time point. In one experiment irrelevant (nonantigen binding) β-lactamase conjugate control was evaluated for LY266070 activation, at drug exposure times of 1 and 3 h. At the 3-h point the ID_{50} values of LY266070 and DAVLBHYD were 0.83 and 0.024 μg/mL, respectively, indicating no enhanced cytotoxicity attributable to the irrelevant conjugate and, therefore, that the activation demonstrated in Figure 5 is an antigen-mediated event.

CONCLUSIONS

A conjugation procedure has been developed that produces a conjugate with the essential properties required for an ADC system. The conjugate has a single binding arm and a molecular weight of about 90 000. Pharmacokinetic studies of antibody fragments in mouse models have shown that mono- and bifunctional Fab'_2 exhibit rapid serum clearance relative to tumor residence in comparison

Table II. Kinetic Parameters for Substrates



	X	Y	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $(s\ \mu M)^{-1}$
KEFLIN	OAc	..[a]	50	5	10
PADAC		..	135	55	2.5
LY191026		..	3300	9	400
LY262319		..	16	2	9
LY262594		O	1000	38	26
LY266070		O	1700	160	11

[a] Electron lone pair.

Table III. Antigen-Dependent β-Lactamase Activity

enzyme	concn, nM	antigen expression	time, min	% Δ[a]
β-l	50	+[b]	10	13[c]
	10	+	10	5
β-lCEM	50	+	10	77
	10	+	10	54
	50	+	1	64
	10	+	1	38
β-lCEM	50	–[d]	10	7
	10	–	10	6

[a] Percent decrease in 570-nm absorbance by PADAC under conditions described in the paper. A large number indicates catalytic degradation of PADAC due to β-lactamase on the cell surface. [b] LS174T cells. [c] A small decrease in absorbance may result from dilution in the assay, uncatalyzed hydrolysis of PADAC, or insufficient washing of the cells. [d] MOLT4 cells.

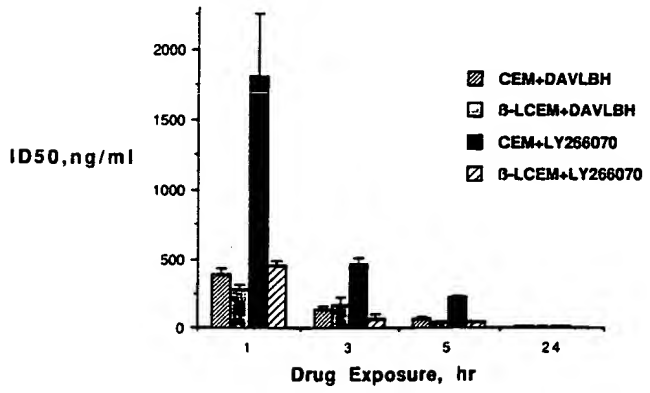


Figure 5. In vitro cytotoxicity was determined as described in the Experimental Procedures. The dose of drug required to inhibit the incorporation of tritiated leucine to 50% of the control value (ID_{50}) is given on the ordinate, while the time of drug exposure is indicated on the abscissa. The data shown in the figure is from a representative experiment. Similar data were obtained in four separate experiments which included drug exposure times of 1, 3, 5, 24, and 48 h. All samples were run in quadruplicate, and the mean ±SD values are indicated in the figure.

to whole antibodies (*35*). Hence, a single binding arm conjugate of less than 100 000 molecular weight shows relatively high tumor to blood concentration ratios at times longer than about 48 h after injection. Furthermore, studies with synthetic bifunctional Fab'_2 have provided evidence that two-step delivery systems can cause localization of small molecules administered independently to antigen-bearing target cells (*1*).

The heterobifunctional cross-linker used for the conjugation, sulfo-SMCC, reacts nonregiospecifically with ε-amino groups of lysine residues on proteins. Since β-l activity was not significantly impaired under the conditions described, it is probable that neither of the lysine residues in the vicinity of the active site of this enzyme are particularly reactive with this reagent. The lack of impact of conjugation on immunoreactivity of the antibody fragment results from the fact that the sulfhydryls available for reaction occur naturally in the hinge region, away from the binding site.

The finding that all of the substrates developed for the ADC delivery system are readily cleaved by the *E. cloacae* β-lactamase (Table II) is in accord with findings of studies on the mechanism of catalyzed and uncatalyzed breakdown of cephalosporins (*15–17, 36–42*) and with the known tolerance of this particular enzyme for substitution of the cephalosporin nucleus. Despite large relativ errors associated with these measurements, it is clear that there is a very wide variation in k_{cat} and K_M and their ratio and that simple substrates, such as Keflin, are not necessarily the most rapidly hydrolyzed. Although this obs rvation is consistent with previous reports (*43–46*), the phenomenon is only poorly understood. Of particular interest is the finding that cephem β-sulfoxides are excellent substrates. Cursory data suggest that they are also more stable to hydrolysis than their native cephem analogues and therefore may be preferred candidates for use in an ADC system.

The conjugate is conveniently assayed using th chromogenic substrate PADAC (*47, 48*). Alternatively, nitrocefin could be used (*49*), but nitrocefin undergoes a spectral change on binding to albumin (*50*) which interferes with its ability to act as a chromogenic substrate. PADAC was chosen so that its use could be applied to samples containing serum.

That the conjugate combined enzymatic activity and antigen binding activity simultaneously was demonstrated

by treating antigen positive cells sequentially with conjugate and PADAC. Cells expressing the antigen clearly exhibited β-lactamase activity, indicating that conjugate was able to perform both the binding and the catalytic function. Negative results in the case of antigen-positive cells treated with native β-lactamase showed that nonspecific interaction of enzyme with cell or antigen was not responsible for the interaction, and negative results in the case of antigen negative cells treated with conjugate illustrated the antibody binding site dependence of the phenomenon.

A reproducible difference in potency between prodrug and drug was observed in leucine incorporation assays on antigen-positive cells in culture. Pretreatment of the cells with conjugate eliminated the differential, thus demonstrating activation of the prodrug, in an antigen-dependent manner. The diminished potency of the prodrug with respect to the drug, and the ability of cell-bound conjugate to activate the prodrug, demonstrated that both prodrug and conjugate had the requisite properties for antibody-directed catalysis. The combination of potency differential and activation was also the primary criterion for selecting candidate prodrugs for further study.

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LITERATURE CITED

(1) Stickney, D. R., Slater, J. B., Kirk, G. A., Ahlem, C. N., Chang, C. H., and Frincke, J. M. (1989) Bifunctional Antibody: ZCE/CHA ^{111}Indium BLEDTA-IV Clinical Imaging in Colorectal Carcinoma. *Antibody Immunoconjugates Radiopharm. 2*, 1–13.

(2) Senter, P. D., Saulnier, M. G., Schreiber, G. J., Hirschberg, D. L., Brown, J. P., Hellström, I., and Hellström, K. E. (1988) Anti-tumor Effects of Antibody–Alkaline Phosphatase Conjugates in Combination with Etoposide Phosphate. *Proc. Natl. Acad. Sci., U.S.A. 85*, 4842–6.

(3) Senter, P. D., Schreiber, G. J., Hirschberg, D. L., Ashe, S. A., Hellström, K. E., and Hellström, I. (1989) Enhancement of the In Vitro and In Vivo Antitumor Activities of Phosphorylated Mitomycin C and Etoposide Derivatives by Monoclonal Antibody-Alkaline Phosphatase Conjugates. *Cancer Res. 49*, 5789–92.

(4) Senter, P. D. (1990) Activation of Prodrugs by Antibody–Enzyme Conjugates: A New Approach to Cancer Therapy. *FASEB J. 4*, 188–93.

(5) Kerr, D. E., Senter, P. D., Burnett, W. V., Hirschberg, D. L., Hellström, I., and Hellström, K. E. (1990) Antibody–Penicillin-V-Amidase Conjugates Kill Antigen Positive Tumor Cells When Combined with Doxorubicin phenoxyacetamide. *Cancer Immunol. Immunother. 31*, 202–6.

(6) Sahin, U., Hartmann, F., Senter, P., Pohl, C., Engert, A., Diehl, V., and Pfreundschuh, M. (1990) Specific Activation of the Prodrug Mitomycin Phosphate by a Bispecific Anti-CD30/Anti-Alkaline Phosphatase Monoclonal Antibody. *Cancer Res. 50*, 6944–8.

(7) Springer, C. J., Antoniw, P., Bagshawe, K. D., Searle, F., Bisset, G. M. F., and Jarman, M. (1990) Novel Prodrugs Which Are Activated to Cytotoxic Alkylating Agents by Carboxypeptidase G2. *J. Med. Chem. 33*, 677–81.

(8) Bagshawe, K. D., Springer, C. J., Searle, F., Antoniw, P., Sharma, S. K., Melton, R. G., and Sherwood, R. F. (1988) A Cytotoxic Agent Can Be Generated Selectively at Cancer Sites. *Br. J. Cancer 58*, 700–3.

(9) Shepherd, T. A., Jungheim, L. N., Meyer, D. L., and Starling, J. J. (1991) A Novel Targeted Delivery System Utilizing a Cephalosporin-Oncolytic Prodrug Activated by an Antibody β-Lactamase Conjugate for the Treatment of Cancer. *BioMed. Chem. Lett. 1*, 21–6.

(10) Lindberg, F., and Normark, S. (1987) Common Mechanism of *ampC* β-Lactamase Induction in Enterobacteria: Regulation of the Cloned *Enterobacter cloacae* P99 β-Lactamase Gene. *J. Bacteriol. 169*, 758–63.

(11) Gootz, T. D., Sanders, C. C., and Goering, R. V. (1982) Resistance to cefamandole: Depression of β-lactamases by cefoxitin and mutation in *Enterobacter cloacae*. *J. Infect. Dis. 146*, 34–42.

(12) Fleming, P. C., Goldner, M., and Glass, D. G. (1963) Observations on the Nature, Distribution, and Significance of Cephalosporinase. *Lancet* 1399–401.

(13) Galleni, M., Lindberg, F., Normark, S., Cole, S., Honore, N., Joris, B., and Frere, J.-M. (1988) Sequence and Comparative Analysis of Three *Enterobacter cloacae ampC* β-Lactamase Genes and their Products. *Biochem. J. 250*, 753–60.

(14) Charlier, P., Dideberg, O., Frere, J.-M., Moews, P. C., and Knox, J. R. (1983) Crystallographic Data for the β-Lactamase from *Enterobacter cloacae* P99. *J. Mol. Biol. 171*, 237–8.

(15) Boyd, D. B., and Lunn, W. H. W. (1979) Electronic Structures of Cephalosporins and Penicillins. 9. Departure of a Leaving Group in Cephalosporins. *J. Med. Chem. 22*, 778–84.

(16) Pratt, R. F., and Faraci, W. S. (1986) Direct observation by ^{1}H NMR of Cephalosporate Intermediates in Aqueous Solution during the Hydrazinolysis and β-Lactamase-Catalyzed Hydrolysis of Cephalosporins with 3′-Leaving Groups: Kinetics and Equilibria of the 3′-Elimination Reaction. *J. Am. Chem. Soc. 108*, 5328–33.

(17) Faraci, S. W., and Pratt, R. F. (1984) Elimination of a Good Leaving Group from the 3′-Position of a Cephalosporin Need Not Be Concerted with β-Lactam Ring Opening: TEM-2 β-Lactamase-Catalyzed Hydrolysis of Pyridine-2-azo-4′-(*N′,N′*-dimethylaniline) Cephalosporin (PADAC) and of Cephaloridine. *J. Am. Chem. Soc. 106*, 1489–90.

(18) Mobashery, S., Lerner, S. A., and Johnston, M. (1986) Conscripting β-Lactamase for Use in Drug Delivery. Synthesis and Biological Activity of a Cephalosporin C10-Ester of an Antibiotic Dipeptide. *J. Am. Chem. Soc. 108*, 1685–6.

(19) Albrecht, H. A., Beskid, G., Christenson, J. G., Durkin, J. W., Fallat, V., Georgopapadakou, N. H., Keith, D. D., Konzelmann, F. M., Lipschitz, E. R., McGarry, D. H., Siebelist, J., Wei, C. C., Weigele, M., and Yang, R. (1991) Dual-Action Cephalosporins: Cephalosporin 3′-Quaternary Ammonium Quinolones. *J. Med. Chem. 34*, 669–75.

(20) Bernier, L. G., Page, M., Gaudreault, R. C., and Joly, L. P. (1984) A Chlorambucil-anti-CEA Conjugate Cytotoxic f r Human Colon Adenocarcinoma Cells In Vitro. *Br. J. Cancer 49*, 245–6.

(21) Rowland, G. F., Simmonds, R. G., Gore, V. A., Marsden, C. H., and Smith, W. (1986) Drug Localization and Growth Inhibition Studies of Vindesine-Monoclonal anti-CEA Conjugates in a Human Tumor Xenograft. *Cancer Immunol. Immunother. 21*, 183–7.

(22) Shih, L. B., and Goldenberg, D. M. (1990) Effects of Methotrexate-Carcinoembryonic-Antigen–Antibody Immunoconjugates on GW-39 Human Tumors in Nude Mice. *Cancer Immunol. Immunother. 31*, 197–201.

(23) Page, M., Delorme, F., Lafontaine, F., and Dumas, L. (1984) Chemotherapy with Daunorubicin–anti-CEA Conjugates in Human Colon Adenocarcinoma Grafted in Nude Mice. *Semin. Oncol. 11*, 56–8.

(24) Iwahashi, T., Tone, Y., Usui, J., Watanabe, H., Sugawara, I., Mori, S., and Okazaki, H. (1989) Selective Killing of Carcinoembryonic-Antigen (CEA)-Producing Cells In Vitr by the Immunoconjugate Cytorhodin-S and CEA-Reactive Cytorhodin-S Antibody CA208. *Cancer Immunol. Immunother. 30*, 239–46.

(25) Corvalan, J. R. F., Smith, W., Gore, V. A., and Brandon, D. R. (1987) Specific In Vitro and In Vivo Drug Localizati n to Tumor Cells Using a Hybrid–Hybrid Monoclonal Antibody Recognizing Both Carcinoembryonic Antigen (CEA) and Vinca Alkaloids. *Cancer Immunol. Immunother. 24*, 133–7.

(26) Corvalan, J. R. F., and Smith, W. (1987) Construction and Characterization of a Hybrid–Hybrid Monoclonal Antibody Recognizing Both Carcinoembryonic Antigen (CEA) and Vinca Alkaloids. *Cancer Immunol. Immunother. 24*, 127–32.

(27) Corvalan, J. R. F. (1988) Tumor Therapy with Vinca Alkaloids Targeted by a Hybrid–Hybrid Monoclonal Antibody Recognizing both CEA and Vinca Alkaloids. *Int. J. Cancer*, Suppl. 2, 22–5.

(28) Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R. (1979) Conjugation of Glucose Oxidase from *Aspergillus niger* and Rabbit Antibodies Using N-Hydroxysuccinimide Ester of N-(4-Carboxycyclohexylmethyl)-Maleimide. *Eur. J. Biochem. 101*, 395–9.

(29) Conrad, R. A., Cullinan, G. J., Gerzon, K., and Poore, G. A. (1979) Structure–Activity Relationships of Dimeric Catharanthus Alkaloids. 2. Experimental Antitumor Activities of N-Substituted Deacetylvinblastine Amide (Vindesine) Sulfates. *J. Med. Chem. 22*, 391–400.

(30) Barnett, C. J., Cullinan, G. J., Gerzon, K., Hoying, R., Jones, W. E., Newlon, W. M., Poore, G. A., Robison, R. L., Sweeney, M. J., and Todd, G. C. (1978) Structure–Activity Relationships of Dimeric Catharanthus Alkaloids. I. Deacetylvinblastine Amide (Vindesine) Sulfate. *J. Med. Chem. 21*, 88–96.

(31) Cartwright, S. J., and Waley, S. G. (1984) Purification of β-Lactamases by Affinity Chromatography on Phenylboronic Acid-Agarose. *Biochem. J. 221*, 505–12.

(32) Cartwright, S. J., and Waley, S. G. (1987) Cryoenzymology of β-Lactamases. *Biochem. 26*, 5329–37.

(33) Beidler, C. B., Ludwig, J. R., Cardenas, J., Phelps, J., Papworth, C. G., Melcher, E., Sierzega, M., Myers, L. J., Unger, B. W., Fisher, M., David, G. S., and Johnson, M. J. (1988) Cloning and High Level Expression of a Chimeric Antibody with Specificity for Human Carcinoembryonic Antigen. *J. Immunol. 141*, 4053–60.

(34) Phelps, J. E., Beidler, D. E., Jue, R. A., Unger, B. W., and Johnson, M. W. (1990) Expression and Characterization of a Chimeric Antibody with Therapeutic Applications. *J. Immunol. 145*, 1200–4.

(35) Mackensen, D. G., Courtney, L. P., Desmond, W. J., Hansen, N., Resch, P. A., Sanders, V. L., Lowe, S. R., Ahlem, C. N., Anderson, L. D., and Frincke, J. M. (1989) Enhanced Antitumor Effect of Multiple Course Radioimmunotherapy Using a Bifunctional Antibody. *J. Nucl. Med. 31* (5th Supplement), 911.

(36) Faraci, W. S., and Pratt, R. F. (1986) Interactions of Cephalosporins with the *Streptomyces* R61 DD-Transpeptidase/Carboxypeptidase. Influence of the 3′-Substituent. *Biochem. J. 238*, 309–12.

(37) Boyd, D. B. (1985) Leaving Group Ability and pK_a in Elimination Reactions. *J. Org. Chem. 50*, 885–6.

(38) Boyd, D. B. (1985) Elucidating the Leaving Group Effect in the β-Lactam Ring Opening Mechanism of Cephalosporins. *J. Org. Chem. 50*, 886–8.

(39) Boyd, D. B. (1984) Electronic Structures of C phalosporins and Penicillins. 15. Inductive Effect of the 3-Position Side Chain in Cephalosporins. *J. Med. Chem. 27*, 63–6.

(40) Boyd, D. B., Hermann, R. B., Presti, D. E., and Marsh, M. M. (1975) Electronic Structures of Cephalosporins and Penicillins. 4. Modeling Acylation by the β-Lactam Ring. *J. Med. Chem. 18*, 408–17.

(41) Page, M. I., and Proctor, P. (1984) Mechanism of β-Lactam Ring Opening in Cephalosporins. *J. Am. Chem. Soc. 106*, 3820–5.

(42) Rao, S. N., and O'Ferrall, R. A. M. (1990) A Structure–Reactivity Relationship for Base-Promoted Hydr lysis and Methanolysis of Monocyclic β-Lactams. *J. Am. Chem. Soc. 112*, 2729–35.

(43) James, R. (1983) Relative Substrate Affinity Index Values: a Method for Identification of Beta-Lactamase Enzymes and Prediction of Successful Beta-Lactam Therapy. *J. Clin. Microbiol. 17*, 791–8.

(44) Labia, R., Morand, A., Tiwari, K., Sirot, J., Sirot, D., and Petit, A. (1988) Interactions of New Plasmid-Mediated β-Lactamases with Third-Generation Cephalosporins. *Rev. Infect. Dis. 10*, 885–91.

(45) Galleni, M., Amicosante, G., and Frere, J.-M. (1988) A Survey of the Kinetic Parameters of Class C β-Lactamases. Cephalosporins and Other β-Lactam Compounds. *Biochem. J. 255*, 123–9.

(46) Mazzella, J., and Pratt, R. F. (1989) Effect of the 3′-Leaving Group on Turnover of Cephem Antibiotics by a Class C β-Lactamase. *Biochem. J. 259*, 255–60.

(47) Schindler, P., and Huber, G. (1980) Use of PADAC, A Novel Chromogenic β-Lactamase Substrate, for the Detection of β-Lactamase Producing Organisms and Assay of β-Lactamase Inhibitors/Inactivators. *Enzyme Inhibitors* (U. Brodbeck, Ed.) pp 169–76, Verlag Chemie, Weinheim.

(48) Thornsberry, C., W. J. Novick, J., and Jones, R. N. (1982) PADAC. Its Use as an Indicator of Relevant Beta-Lactamase Production In Clinical Bacterial Isolates. Calbiochem-Behring product brochure, 10933 N. Torrey Pines R., La Jolla, CA, 92037.

(49) O'Callaghan, C. H., Morris, A., Kirby, S. M., and Shingler, A. H. (1972) Novel Method for Detection of β-Lactamase by Using a Chromogenic Cephalosporin Substrate. *Antimicrob. Ag. Chemother. 1* (4), 283–8.

(50) Jones, R. N., Wilson, H. W., and W. J. Novick, J. (1982) In Vitro Evaluation of Pyridine-2-azo-*p*-dimethylaniline Cephalosporin (PADAC), a New Diagnostic Chromogenic Reagent Compared to Nitrocefin, Cephacetrile, and Other B ta-Lactam Compounds. *J. Clin. Microb. 15*, 954–8.

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